MYCOTAXON
ISSN (print) 0093-4666 (online) 2154-8889 Mycotaxon, Ltd. ©2017
October–December 2017—Volume 132, pp. 799–811
https://doi.org/10.5248/132.799

Amanita pallidorosea in Pakistan and its ectomycorrhizal association with *Quercus oblongata*

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Abstract—The occurrence of *Amanita pallidorosea* in Pakistan and its ectomycorrhizal association are reported for the first time based on morphological characters and nucleotide sequences of the internal transcribed spacer region generated from basidiomata and from ectomycorrhizal roots of *Quercus oblongata* [= *Q. incana* Roxb., nom. illeg.].

Key words—*Amanitaceae*, ITS, lethal, phylogeny, Swat

Introduction

Amanita Pers. (*Agaricales, Basidiomycota*) is a large genus comprising about 500 described species (Bas 2000, Tulloss 2005, Yang 2005, Kirk et al. 2008). Many *Amanita* species have been shown to be ectomycorrhizal, thus playing vital roles in forest ecology (Yang 1997). New *Amanita* species have been regularly reported from all over the world, including East Asia, Central and South America, South Africa, and Australia (Zhang et al. 2015). However, knowledge about the genus is rather limited for Pakistan, where only eighteen species have been reported (Ahmad et al. 1997, Tulloss et al. 2001). The goal of this study was to collect *Amanita* basidiomata and ectomycorrhizae from the *Quercus*-dominated forests of Swat district, Khyber Pakhtunkhwa province, Pakistan. Mycological expeditions took place during the monsoon season (July to September) of 2015. Nuclear ribosomal DNA sequence analyses of the ITS region from basidiomata and ectomycorrhizal roots were combined with detailed morphological examinations to confirm Pakistani collections as *A. pallidorosea,* a new record for Pakistan. We describe the ectomycorrhiza of this species here for the first time.

Materials & methods

Sampling site

Different sites dominated by oak forests were selected for sampling within district Swat (34°34′–35°55′N 72°08′–72°50′E). The Swat valley, well known for its biodiversity, is in Pakistan's Khyber Pakhtunkhwa (KP) province (Shinwari et al. 2003) where altitudes range from 970 m to 2300 m (Barth 1956), with Falak Sar (the highest peak) towering 5918 m above sea level. The district's climate is dry temperate to moist temperate. Most of the region gets monsoon rains in summer except in the north, which receives very little or no summer monsoon rains (Ali & Qaisar 1986, Ahmad & Sirajuddin 1996). Swat vegetation types range from sub-tropical chir pine (*Pinus roxburghii* Sarg.) forest in the south to alpine pastures and meadows or cold desert in the north (Sher 2013).

Collection and morphological characterization of the basidiomata

Basidiomata were collected, tagged and photographed in the field using a Nikon D70S digital camera. Fresh morphological characters were recorded in the field and colors were designated using Munsell (1975) after which the specimens were dried with the help of a fan heater and sealed in zip-lock bags for long-term preservation. The dried material was vouchered and deposited in the Lahore Herbarium, Department of Botany, University of the Punjab, Pakistan (LAH).

Microscopic characteristics were observed in 5% KOH solution. The abbreviation 'n/b/p' indicates 'n' basidiospores measured from 'b' basidiomata from 'p' collections. Basidiospore dimensions are given as length × width (l × w), with extreme values given in parentheses, and Q = l/w ratio of individual spores; avQ = average Q of all spores ± standard deviation (Zhang et al. 2010).

Isolation and morphological characterization of ectomycorrhizal roots

Twenty 15 cm^3 soil cores near the soil surface were retrieved from each sampling site close enough to *Quercus* tree trunks to ensure that they contained fine *Quercus* roots. Each soil core was later soaked in water in the laboratory for 1–2 hrs and then passed over a 2 mm sieve under running water to separate the roots from soil. Ectomycorrhizal roots were carefully sorted into morphotypes using a Meiji Techno EMZ-5TR stereomicroscope. The fresh ectomycorrhizal roots were cleaned with a fine camel hair brush, photographed under the stereomicroscope, and examined anatomically under a compound microscope. The morphotypes were kept in McCartney bottles in distilled water with replicates of each reserved in 2% CTAB buffer at 8°C for molecular analysis.

DNA extraction, PCR and sequencing

DNA was extracted (from 3–4 gills or 0.5–1 mg of context tissue per basidioma or 2–4 ectomycorrhizal root tips per morphotype) using a modified CTAB method (Bruns 1995). The extracted genomic DNA was evaluated using gel electrophoresis (1% agarose gel in Gel documentation system Uvipro Platinum (IM-2000 WL/LC/26M MANZ) with default settings. After confirmation, genomic DNA was suspended in nuclease free water and stored at 20°C. The target rDNA internal transcribed spacer region (ITS1 + 5.8S + ITS2) was amplified by optimized polymerase chain reaction (PCR) (Gardes & Bruns 1993) using the ITS1F fungal specific forward primer (5′–CTTGGTCATTTAGAGGAAGTAA–3′; Gardes & Bruns 1993), ITS4 reverse primer (5′–TCCTCCGCTTATTGATATGC–3′; White et al. 1990), and TAQ polymerase as catalyst in 20 µL PCR tubes using Gene Amp PCR system 9700. The thermo-cycling profile had an initial and final denaturation step at 94°C for 1 minute followed by 30 cycles at 53°C for 1 minute for annealing, and a 1-minute initial extension and 8-minute final extension at 72°C. PCR products were visualized by using 1% agarose gel. Amplified products were sequenced in both directions with the ITS1F and ITS4 amplification primers by BGI Tech Solutions (HK Co. Ltd.). To identify the plant host species, the host plant DNA was extracted from root tips and amplified using the plant specific primer 28KJ (5′–GGCGGTAAATTCCGTCC–3′) and universal primer 28C (5′–GCTATCCTGAGGGAAACTT–3′) (Cullings 1992). Successful amplicons obtained from ectomycorrhizal roots were purified using Exonuclease I and Shrimp Alkaline Phosphatase enzymes (Werle et al. 1994). The purified products were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology Research (http: //www. biotech. ufl.edu/). Sequence chromatograms were trimmed, edited, and assembled using Sequencher 4.1 (Gene Codes, Ann Arbor, MI).

Sequence alignment and phylogenetic analysis

Consensus sequences were generated from the obtained sequences with the forward and reverse algorithms in BioEdit (Hall 1999) and then BLAST searched at NCBI (http://www.ncbi.nlm.nih.gov/). Closely matched sequences from GenBank were downloaded for phylogenetic analysis based on BLAST analysis and on sequences generated by previous phylogenetic studies of *Amanita pallidorosea* (Zhang et al. 2010, Kim et al. 2013, Li et al. 2015). Sequences were manually edited and assembled using BioEdit (Hall 1999) and ClustalW (Thompson et al. 1997). All ITS sequences were trimmed with the conserved motifs 5′–(...GAT)CATTA– and –GACCT(CAAA...)–3′ to facilitate alignment, with the the alignment portion between them included in the analysis (Dentinger et al. 2011). These sequences were aligned with other GenBank sequences using the MUSCLE (Edgar 2004) alignment tool available online (www.ebi.ac.uklTools/msalmuscle). In aligned dataset, all characters were equally weighted, and gap positions were treated as missed data. A phylogenetic tree was constructed with the maximum likelihood (ML) analysis using Jukes-Cantor model in MEGA6 software to test the phylogeny (Tamura et al. 2011). All obtained sequences have been submitted to and accessioned by GenBank.

Results

Amanita pallidorosea P. Zhang & Zhu L. Yang,
Fungal Diversity 42: 125 (2010) FIGS 1, 2

BASIDIOMATA medium sized. PILEUS 40–60 mm diam., convex to plano-convex, slightly umbonate, the disc pale pink (5YR9/4) to pale rose (10R9/6) and becoming white with maturity or under dry conditions; surface dry, smooth, volval remnants sometimes present, dull to shiny, margins smooth, incurved when young becoming straight and splitting with age or in dry conditions; context dry, whitish or slightly paler than cap, 1–3 mm, texture soft, not changing upon cutting or bruising. ODOR not distinctive; taste not recorded. LAMELLAE 4.5 mm broad, free, crowded, whitish at first then turning creamy to yellowish cream (10YR9/8) at maturity, edge entire. LAMELLULAE attenuate, frequent, length variable. STIPE 80–120 × 6–12 mm, central, cylindrical to sub-cylindrical to tapering upwards, white to creamy white above the annulus and creamy white with a yellowish (10YR9/8) to yellowish pink (10R7/10) tinge below, upper quarter hollow, below solid; surface smooth to finely fibrillose squamulose; context white. ANNULUS present, membranous, thin, permanent, yellow to white. VOLVA saccate, white, membranous, permanent, in some specimens breaking to leave fragments on the stipe, giving the stipe a banded appearance.

BASIDIOSPORES [n/b/p = 80/4/3] 7–8.5(–9) × (6.5–)7–8.5(–9) µm, Q = 1–1.08, avQ = 1.01, globose to subglobose, apiculus prominent (1.5 µm long), guttulate, amyloid in Melzer's reagent. BASIDIA 31–40 × 8–10.5 µm, clavate, tetra-sterigmate, basal clamps absent. PILEIPELLIS hyphae 3.5–5(–7.5) µm diam., filamentous, terminal inflated cells clavate 15 µm diam., hyaline; when present, volval remnant cells abundant, inflated (18.5 × 18 µm). STIPITIPELLIS filamentous, hyaline, 4.5–12 µm diam. VOLVAL CELLS subglobose to oval (66 × 78.5 µm), hyphae 5–7 µm diam. CLAMP CONNECTIONS absent in all tissues.

SPECIMENS EXAMINED: **PAKISTAN, KHYBER PAKHTUNKHWA PROVINCE, Swat District**, Lower Shawar, 2100 m asl, on ground in a forest dominated by *Quercus oblongata*, 5 September 2015, Zia-ullah Sh03 (LAH35204; GenBank KY626178); Malam Jabba, Kishawra, 2200 m asl, on soil under oak, 16 August 2015, Junaid Khan MJ1554 (LAH35205; GenBank KY621476); Toa, 2800 m asl, on ground under *Quercus oblongata*, 15 July 2015, Arooj Naseer T4 (LAH35206; GenBank KY616968).

COMMENTS: Macroscopically, *Amanita subpallidorosea* Hai J. Li is strikingly similar to *A. pallidorosea* because of its pale rose pileus with a conspicuous umbo. However, microscopically, *A. subpallidorosea* can be distinguished from *A. pallidorosea* by its larger basidia and basidiospores (Li et al. 2015, Yang 2015). Pure white specimens of *A. pallidorosea*, also common (Zhang et

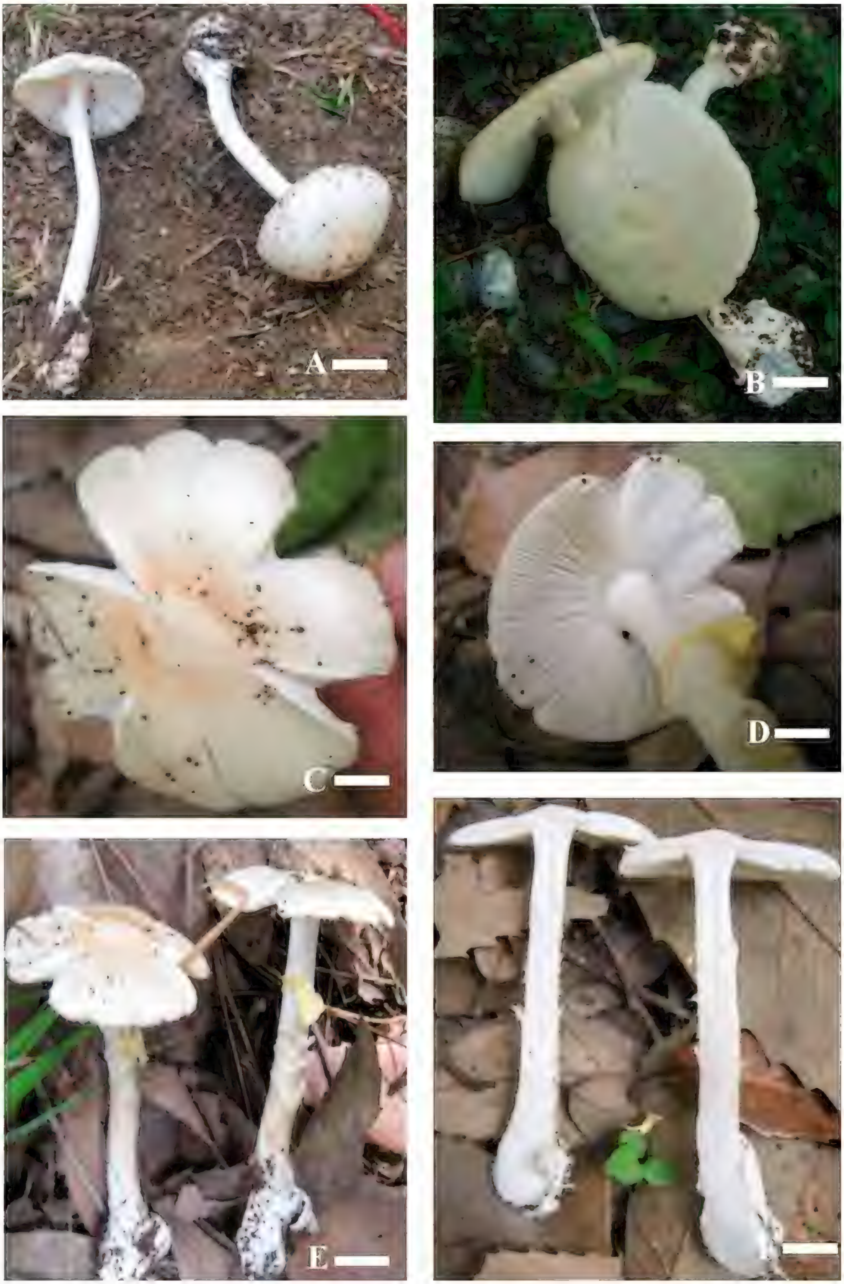

FIG. 1. *Amanita pallidorosea* basidiomata. A. LAH35204; B. LAH35206; C–F. LAH35205. Scale bars: A = 1.9 cm; B =1.6 cm; C = 0.9 cm; D = 1.1 cm; E = 1.7 cm; F = 1.5 cm.

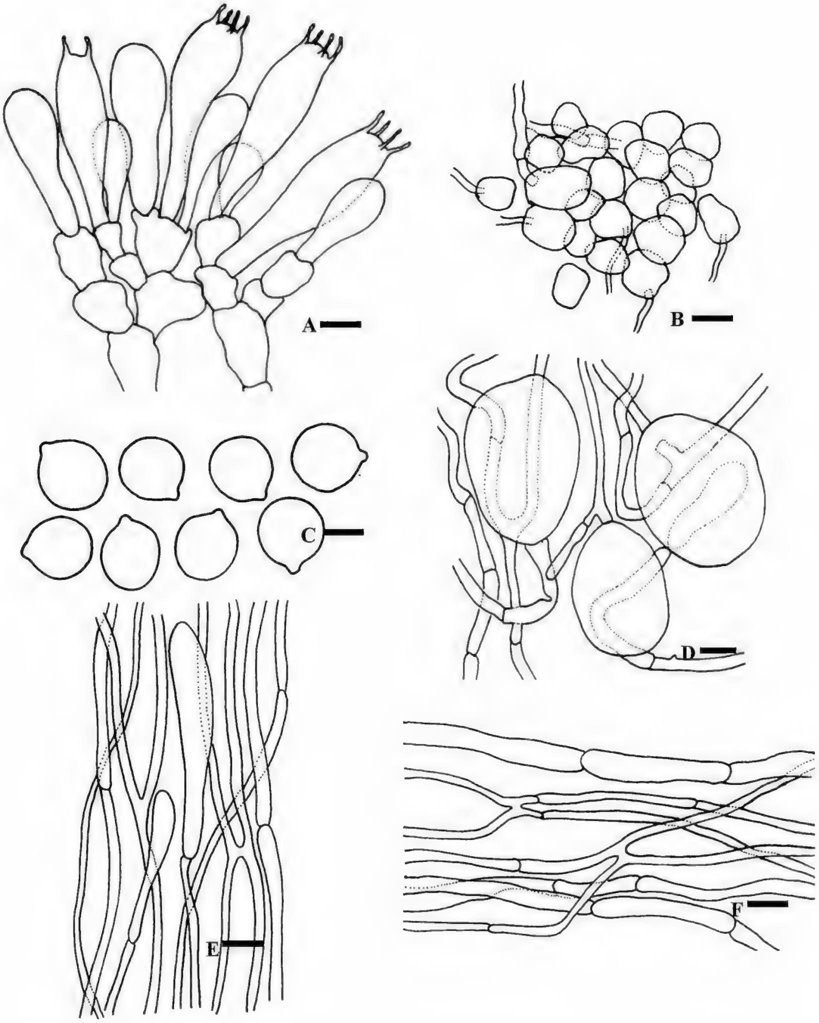

Fig. 2. *Amanita pallidorosea* (LAH35204). A. basidia and subhymenium; B. volval remnants on pileus; C. basidiospores; D. volva; E. pileipellis; F. stipitipellis. Scale bars: A = 7.5µm; B = 30 µm; C = 6.5 µm; D = 25 µm; E, F = 20 µm. Drawings by M. Kiran.

al. 2010, Yang 2015), can be easily confused with *A. virosa* Bertill. (Malysheva et al. 2014). *Amanita virosa*, however, can be distinguished with its distinctly recurved squamules on the stipe and larger basidiospores (Neville & Poumarat 2004, Yang 2015).

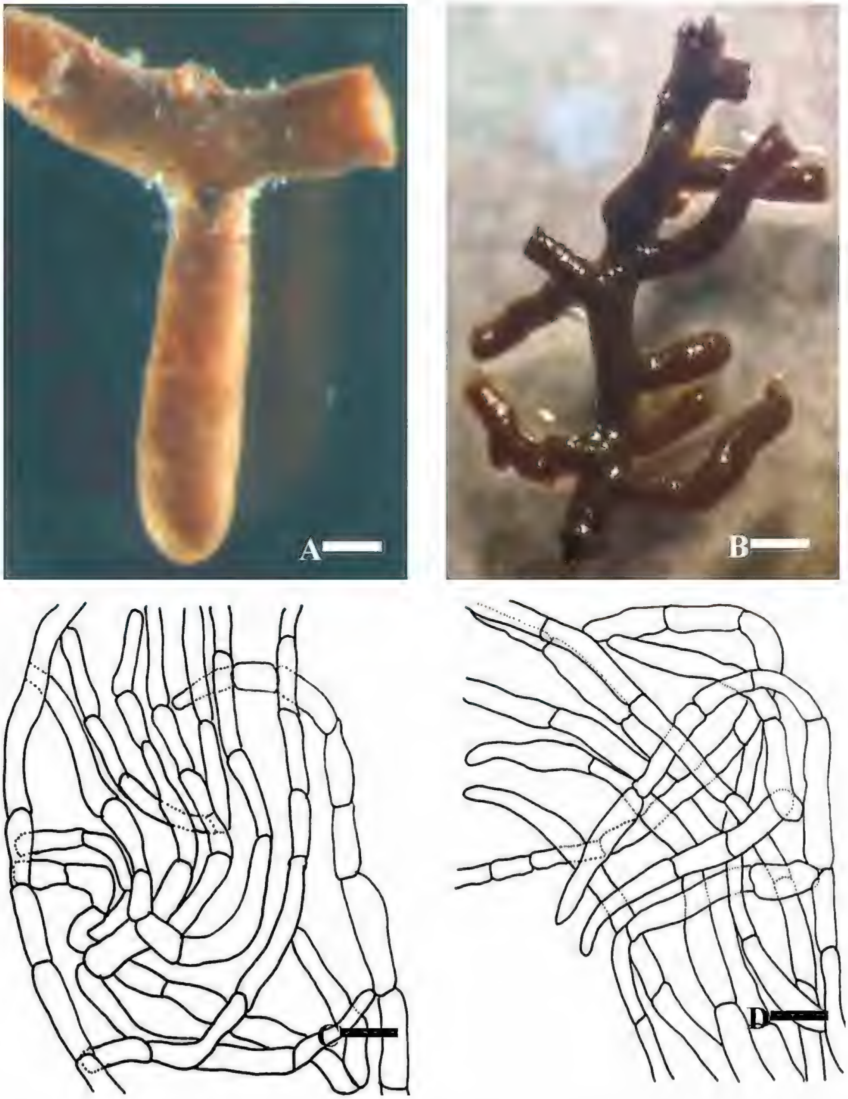

FIG. 3. *Amanita pallidorosea/Quercus oblongata* mycorrhiza (LAH-EM64). A, B. ectomycorrhizal roots; C. inner mantle layer cells; D. outer mantle layer cells. Scale bars: A = 0.6 mm; B = 1.2 mm; C = 7.5 μm; D = 10 μm. Drawings by M. Kiran.

Morphological characterization of ectomycorrhizae FIG. 3

ECTOMYCORRHIZAL SYSTEM dichotomous, 6–18 mm long, axis ≤1 mm diam., unramified, ends straight, cylindrical, surface smooth, dark brown

(10R1/4). OUTER MANTLE hyphal cells 4.39–5.85 µm diam., loosely arranged. INNER MANTLE hyphal cells 4.17–4.80 µm compactly arranged. Cells in both layers are mantle type A (Agerer 1987–2002), frequently septate, branched, thin-walled, yellowish brown, end cells not sharply pointed. EMANATING HYPHAE rarely present. RHIZOMORPHS not observed.

HABITAT & DISTRIBUTION: In Pakistan, solitary to scattered in small groups on ground under oak. Originally described from China (Zhang et al. 2010); also known from Japan, Korea, and eastern Russia (Kim et al. 2013, Malysheva et al. 2014, Yang 2015, Cai et al. 2016).

SPECIMENS EXAMINED: **PAKISTAN, KHYBER PAKHTUNKHWA PROVINCE, Swat District**, Toa, 2800 m asl, associated with roots of *Quercus oblongata*, 15 July 2015, Arooj Naseer ANT58 (LAH-EM65; GenBank KY616969); 15 July 2015, Arooj Naseer ANT81 (LAH-EM66; GenBank KY616970); 15 July 2015, Arooj Naseer ANT450044 (LAH-EM67; GenBank KY616971); Shawar, 2100 m asl, on roots of *Quercus oblongata*, 14 August 2015, Arooj Naseer AN26 (LAH-EM64; GenBank KY616967).

Molecular phylogenetic characterization FIG. 4

The host plant sequence (GenBank KY829452) obtained from ectomycorrhizal roots of *Amanita pallidorosea* (GenBank KY616969) showed 99% identity with *Quercus oblongata* D. Don [= *Quercus incana* Roxb., nom. illeg.] (GenBank AY456170) using BLAST search tool at NCBI. Phylogenetic analysis was performed on a 58-sequence ITS dataset of which 57 sequences represented *Amanita* species and one represented the outgroup *Limacella glioderma* (Fr.) Maire [= *L. delicata* (Fr.) Konrad & Maubl.]. The post-alignment dataset included 891 sites, of which 339 were conserved, 489 variable, and 345 parsimony informative. Reliability of the consensus tree was calculated by 1000 bootstrap replications. The phylogenetic tree contained four well supported clades corresponding to four *Amanita* sections: *A.* sect. *Phalloideae*, *A.* sect. *Validae*, *A.* sect. *Lepidella*, and *A.* sect. *Amanita* (Zhang et al. 2010, Cai et al. 2016). Sequences obtained from the Pakistani basidiomata and morphotypes clustered with *A. pallidorosea* in A. sect. *Phalloideae.* This lineage is consistent with the morphological identification.

Discussion

Amanita pallidorosea is one of the lethal amanitas in *A.* sect. *Phalloideae* (Zhang et al. 2010, Malysheva et al. 2014, Yang 2015). First described by Zhang et al. (2010) from China, the species is characterized by typically pale rosy (sometimes white) umbonate pileus and small globose to subglobose basidiospores. This taxon has been reported from other Asian countries

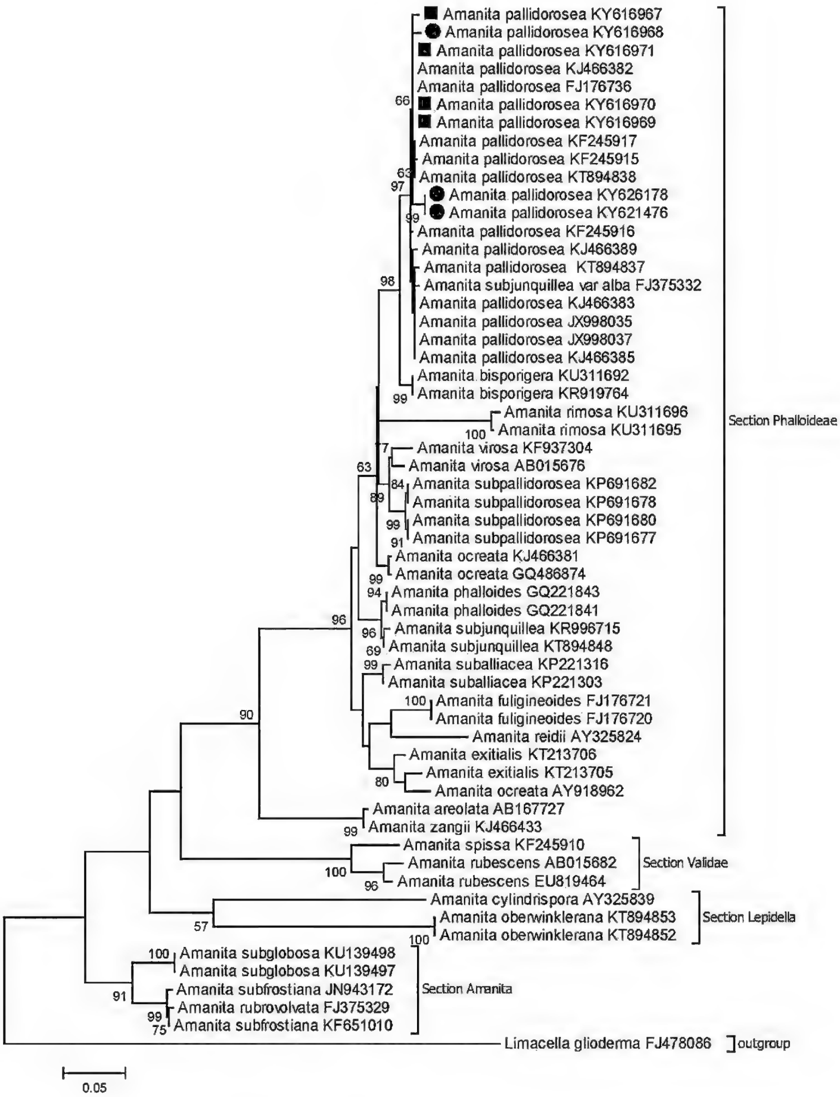
■ Amanita pallidorosea KY616967
● Amanita pallidorosea KY616968
■ Amanita pallidorosea KY616971
Amanita pallidorosea KJ466382
Amanita pallidorosea FJ176736
66
■ Amanita pallidorosea KY616970
■ Amanita pallidorosea KY616969
Amanita pallidorosea KF245917
Amanita pallidorosea KF245915
63
97
Amanita pallidorosea KT894838
● Amanita pallidorosea KY626178
99
● Amanita pallidorosea KY621476
Amanita pallidorosea KF245916
Amanita pallidorosea KJ466389
Amanita pallidorosea KT894837
98
Amanita subjunquillea var alba FJ375332
Amanita pallidorosea KJ466383
Amanita pallidorosea JX998035
Amanita pallidorosea JX998037
Amanita pallidorosea KJ466385
Amanita bisporigera KU311692
99
Amanita bisporigera KR919764
Amanita rimosa KU311696
100
Amanita rimosa KU311695
77
Amanita virosa KF937304
63
Amanita virosa AB015676
84
Amanita subpallidorosea KP691682
89
Amanita subpallidorosea KP691678
99
Amanita subpallidorosea KP691680
91
Amanita subpallidorosea KP691677
Amanita ocreata KJ466381
99
Amanita ocreata GQ486874
94
Amanita phalloides GQ221843
96
Amanita phalloides GQ221841
96
Amanita subjunquillea KR996715
69
Amanita subjunquillea KT894848
99
Amanita suballiacea KP221316
Amanita suballiacea KP221323
100
Amanita fuligineoides FJ176721
90
Amanita fuligineoides FJ176720
Amanita reidii AY325824
Amanita exitialis KT213706
80
Amanita exitialis KT213705
Amanita ocreata AY918962
Amanita areolata AB167727
99
Amanita zangii KJ466433
Section Phalloideae
Amanita spissa KF245910
Amanita rubescens AB015682
100
96
Amanita rubescens EU819464
Section Validae
Amanita cylindrispora AY325839
57
Amanita oberwinklerana KT894853
100
Amanita oberwinklerana KT894852
Section Lepidella
100
Amanita subglobosa KU139498
Amanita subglobosa KU139497
91
Amanita subfrostiana JN943172
Amanita rubrovolvata FJ375329
99
75
Amanita subfrostiana KF651010
Section Amanita
Limacella glioderma FJ478086
outgroup
0.05

Fig. 4. Molecular phylogenetic analysis of *Amanita* spp. based on ITS sequences. The tree with the highest log likelihood (–5886.3380) is shown. The analysis involved a total of 58 nucleotide sequences and 891 positions in the final dataset. New sequences generated from Pakistani basidiomata are marked with ● and from ectomycorrhizal root tips with ■.

including Japan (Yang 2015, Cai et al. 2016), Korea (Kim et al. 2013, Cho et al. 2015), and Russia (Malysheva et al. 2014). Macro- and microscopically, the three *A. pallidorosea* basidiomata (LAH35204–35206) collected in Pakistan agree with the descriptions by Zhang et al. (2010) and Malysheva et al. (2014). However, the smooth stipe surface in some specimens and the hollow stipe apex contrast with the original description. The yellowish annulus in some collections, although differing from the original collection of Zhang et al. (2010), conforms to the findings of Malysheva et al. (2014) and Yang (2015). *Amanita pallidorosea* has not been previously recorded from Pakistan (Ahmad et al. 1997), and our basidiomata and ectomycorrhizal roots represent the first report of the species from the country. Ectomycorrhizal association of *Amanita muscaria* (L.) Lam., *A. citrina* Pers., and *A. strobiliformis* (Vittad.) Bertill. have been described with various hosts with different ramification patterns (Ingleby et al. 1990, Mleczko 2004, Raidl & Verma 2006)

Phylogenetically, the sequences obtained from ectomycorrhizal roots and basidiomata clustered with *A. pallidorosea* sequences retrieved from GenBank in clade sect. *Phalloideae*, which contained *A. areolata* T. Oda et al., *A. bisporigera* G.F. Atk., *A. exitialis* Zhu L. Yang & T.H. Li, *A. fuligineoides* P. Zhang & Zhu L. Yang, *A. ocreata* Peck, *A. phalloides* (Fr.) Link, *A. reidii* Eicker & Greuning, *A. rimosa* P. Zhang & Zhu L. Yang, *A. suballiacea* (Murrill) Murrill, *A. subjunquillea* S. Imai, *A. subjunquillea* var. *alba* Zhu L. Yang, *A. subpallidorosea*, *A. virosa*, and *A. zangii* Zhu L. Yang et al. (Cai et al. 2014, 2016). Large white basidiomata morphologically similar to *A. pallidorosea* are also present in *A. bisporigera*, *A. exitialis, A. oberwinkleriana* Zhu L. Yang & Yoshim. Doi, *A. ocreata*, *A. subjunquillea* var. *alba*, and *A. virosa* (Imazeki & Hongo 1987, Yang & Li 2001, Malysheva et al. 2014) (Fig. 4).

Amanita pallidorosea has been previously reported from forests dominated by *Quercus mongolica* Fisch. ex Ledeb., and members of family *Fagaceae* and *Pinaceae* (Zhang et al. 2010, Malysheva et al. 2014, Cai et al. 2016), but no studies have been published confirming its ectomycorrhizal association. This appears to be the first report of ectomycorrhizal association of *A. pallidorosea* with *Quercus oblongata* supported by morphological and molecular data.

Acknowledgements

The authors are grateful to Prof. Zhu Liang Yang (Kunming Institute of Botany, Chinese Academy of Sciences, China) for his valuable comments and suggestions to improve the manuscript. Thanks are due to Dr. Sana Jabeen (University of Education, Faisalabad Campus, Pakistan), Dr. Andrew W. Wilson (Denver Botanic Gardens, USA), and Dr. Yan-Chun Li (Kunming Institute of Botany, Chinese Academy of Sciences, China) for providing helpful reviews of this manuscript. We very much appreciate the field assistance of Dr. Abdul Rehman Khan Niazi (University of the Punjab, Lahore, Pakistan). Sincere thanks are also due to Dr. Matthew E. Smith (University of Florida, USA) for providing molecular research facilities to one of the authors. Thanks are also due to Dr. Shaun Pennycook for his precious time in editing the nomenclature.

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